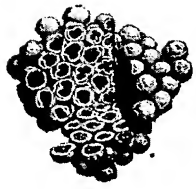

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Original Article

Cell Surface and Transcriptional Characterization of Human Adipose-Derived Adherent Stromal (hADAS) Cells

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Key Words. Adult stem cells • Flow cytometry • Microarray • Integrins

ABSTRACT

Adult human subcutaneous adipose tissue contains cells with intriguing multilineage developmental plasticity, much like marrow-derived mesenchymal stem cells. Putative stem or progenitor cells from fat have been given many different names in the literature, reflecting an early and evolving consensus regarding their phenotypic characterization. The study reported here used microarrays to evaluate over 170 genes relating to angiogenesis and extracellular matrix in undifferentiated, early-passage human adipose-derived adherent stromal (hADAS) cells isolated from three separate donors. The hADAS populations unanimously transcribed 66% of the screened genes, and 83% were transcribed by at least two of the three populations. The most highly transcribed genes relate to functional groupings such as cell adhesion, matrix proteins, growth factors and receptors, and proteases. The transcriptome of hADAS cells demonstrated by this work reveals many similarities to published profiles of bone marrow mesenchymal stem cells (MSCs). In addition, flow analysis of over 24 hADAS cell surface proteins (n = 7 donors) both confirms and expands on the existing literature and reveals strong intergroup correlation, despite an inconsistent nomenclature and the lack of standardized protocols for cell isolation and culture. Finally, based on flow analysis and reverse transcription polymerase chain reaction studies, our results suggest that hADAS cells do not express several proteins that are implicated as markers of "stemness" in other stem cell populations, including telomerase, CD133, and the membrane transporter ABCG2.
STEM CELLS *2005;23:412–423*

INTRODUCTION

Adult stem cells hold great promise for use in tissue repair and regeneration. In recent years, interest has rapidly grown in the developmental plasticity and therapeutic potential of stromal cells that have been isolated from human subcutaneous adipose tissue. Adipose tissue represents an abundant, practical, and appealing source of donor tissue for autologous cell replacement.

Several groups have demonstrated that mesenchymal cells within the stromal-vascular fraction (SVF) of subcutaneous adipose tissue display multilineage developmental plasticity in vitro and in vivo [1–6]. These cells have alternatively been referred to as processed lipoaspirate cells (PLA), adipose-derived stem cells, adipose-derived stromal cells, and adipose-derived mesenchymal progenitor cells. It is also likely that cells previously considered preadipocytes are essentially the same cell population. These many names reflect a lack of consensus and an evolving knowledge base

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with regard to the anatomic origin, phenotype, and function of these cells. Our lab has chosen to refer to these cells as adipose-derived adherent stromal (ADAS) cells. This is a purely descriptive name that also provides some distinction from stromal vascular fraction (SVF) cells, which have not been further separated based on adherence to tissue culture plastic.

There are obvious similarities between hADAS cells and mesenchymal stem cells (MSCs). Both represent the stromal cell fraction isolated from an adipose depot (subcutaneous tissue for the former, bone marrow for the latter) on the basis of adherence to tissue culture plastic. While an extensive body of work exists pertaining to the phenotypic characterization of MSCs, the phenotypic characterization of hADAS cells is in its infancy [7–12]. The plasticity observed of cultured hADAS cells is intriguing and includes cell lineages thought of as ectodermal and mesodermal.

There are no known stem cell–specific markers for the prospective identification of putative stem cells or progenitor cells within adipose tissue, bone marrow, or any other mesodermally derived adult tissue. Based on extensive work with other stem cell populations, however, several proteins have emerged as candidate markers associated with a primitive, stem cell phenotype; these include telomerase, CD133, and ABCG2) [13–18]. The purpose of this study was threefold: (1) to further characterize early passage, undifferentiated hADAS cells on a transcriptional and cell surface level, (2) to determine the degree of variability of cell populations isolated from different donors, and (3) to determine whether hADAS cells specifically exhibit markers that are associated with other stem cell populations.

Materials and Methods

Cell Culture

Delbucco's modified Eagle's medium (DMEM/F12) (cat. no. 11320-033), 0.5% trypsin-EDTA (cat. no. 15400-054), and antibiotic-antimycotic (cat. no. 15240-062) are from Gibco-Invitrogen Corp. (Grand Island, NY; http://www.invitrogen.com). Fetal bovine serum (FBS) (cat. no. F-2442) is from Sigma Chemical Corp. (St. Louis, http://www.sigma-aldrich.com). Culture dishes are from NUNC Brand Products (Nalge Nunc Int., Rochester, NY, http://www.nuncbrand.com/). Liberase Blendzyme 1 is from Roche (Indianapolis, IN; http://www.roche-applied-science.com).

Antibodies

See Table 1 for a full list of antibodies used and their commercial sources.

Table 1. Antibodies and vendors used in the present study

Ab	Isotype	Vendor
PE anti-human HLA-ABC	PE mouse IgG2a	eBioscience
PE anti-human HLA-DR	PE mouse IgG2b	eBioscience
PE anti-human CD4	PE mouse IgG1	eBioscience
PE anti-human CD8a	PE mouse IgG1	eBioscience
PE anti-human CD11c	PE mouse IgG1	eBioscience
PE anti-human CD62L	PE mouse IgG1	eBioscience
PE anti-human CD62P	PE mouse IgG1	eBioscience
PE anti-human CD117	PE mouse IgG1	eBioscience
PE anti-human CD138	PE mouse IgG1	eBioscience
PE anti-human CD11a	PE mouse IgG1	eBioscience
PE anti-human CD11b	PE mouse IgG1	eBioscience
PE anti-human CD49d	PE mouse IgG1	eBioscience
PE anti-human CD106	PE mouse IgG1	eBioscience
PE anti-human CD18	PE mouse IgG1	Research Diagnostics Inc. (RDI)
PE anti-human CD29	PE mouse IgG1	RDI
PE anti-human CD41a	PE mouse IgG1	RDI
PE anti-human CD49b	PE mouse IgG1	RDI
PE anti-human CD51	PE mouse IgG1	RDI
PE anti-human CD61	PE mouse IgG1	RDI
PE anti-human CDw90 Thy.1	PE mouse IgG1	RDI
PE anti-human CD49e	PE mouse IgG2b	RDI
PE anti-human CD49f	PE mouse IgG2b	RDI
FITC anti-human CD103	FITC mouse IgG2a	RDI
PE anti-human CD140a (PDGFRa)	PE mouse IgG2a	BD Biosciences
PE anti-human CD133	PE mouse IgG1	Mitenyi Biotech.
PE anti-human CD243 (MDR-1)	PE mouse IgG1	Chemicon Int.
ABCG2	PE mouse IgG 2b	Santa Cruz Biotech.

Abbreviations: FITC, fluorescein isothiocyanate; IgG, immunoglobulin, PE, phycoerythrin.

Cell Isolation and Culture

Subcutaneous adipose tissue was obtained from patients undergoing elective surgical procedures in the Department of Plastic Surgery, University of Virginia. The University of Virginia's Human Investigation Committee approved tissue harvest protocols. All adipose tissue came either from intraoperative suction lipectomy or from laboratory liposuction of panniculectomy specimens.

Cells were isolated from adipose tissue using methods previously described [1, 19]. Briefly, harvested tissue was washed several times and then enzymatically dissociated. The dissociated tissue was then filtered to remove debris, and the resulting cell suspension was centrifuged. Pelleted stromal cells were then recovered and washed several times. Contaminating erythrocytes were lysed with an osmotic buffer, and the stromal cells were plated onto tissue culture plastic (10-cm dishes). Cultures were washed with buffer 24–48 hours after plating to remove unattached cells, and then refed with fresh medium. Plating and expansion medium consisted of DMEM/F12 with 10% FBS and antibiotic-antimycotic. Cultures were maintained at 37°C with 5% CO_2 and fed three times per week.

Cells were grown to confluence after the initial plating (P = 0), typically within 10–14 days. Once confluent, the adherent cells were released with 0.5% trypsin-EDTA and then either re-plated at 2 k/cm^2 or used for experimental analysis. Cultures were passaged every 7–8 days until they were ready for analysis. All cells used for analysis were considered early passage (passage 2 or earlier), which corresponded to approximately six or fewer total population doublings.

Representative samples of each cell population were evaluated for multilineage developmental plasticity using in vitro assays well described in the literature [1, 5, 20]. Differentiation along the adipogenic, osteogenic, and neurogenic lineages was assessed qualitatively based on cell morphology, histochemistry (Oil Red O or von Kossa stain), or immunohistochemistry (TUJ1).

Gene Array Analysis

Total RNA was isolated from 1.6×10^6 adipo-derived stromal cells obtained from each of three separate donors (age 30–47 years; mean 40 years). The RNA was isolated using the UltraSpec-II RNA Kit (Biotecx Laboratories, Inc., Houston, TX; http://www.biotecx.com/), according to manufacturer's instructions. RNA was measured by spectroscopy with an A_{260}/A_{280} ratio of 1:8. Isolated RNA was used to evaluate the transcription profile of a variety of angiogenic and extracellular matrix factors within each donor cell population using commercially available gene arrays (SuperArray Bioscience Corp., Bethesda, MD; http://www.superarray.com/). RNA was standardized among patients and samples by concentration. The gene array assays were performed according to manufacturer's instructions. Briefly, biotinylated cDNA probes were generated from 3.92 μg of total RNA using gene-specific sets of primers for reverse transcription. The cDNA probes were then hybridized with gene-specific cDNA fragments and spotted on nylon membranes overnight at 68°C. After washing, the arrays were exposed to X-ray film and visualized by autoradiography. The relative expression level of each gene was analyzed using the GEArray Analyzer (SuperArray Bioscience). Each array included several positive controls (cyclophilin-A, β-actin) and a bacterial plasmid (PUC18) as a negative control. A gene was considered to be expressed if its relative expression was greater than that of the negative control PUC18, which served as a proxy measure for background signal. Results were checked against gross evaluation of the X-ray film. Expression levels of select genes were then calculated relative to the positive control gene cyclophilin-A, which had the lowest expression threshold of all of the positive controls.

Flow Cytometry

Early passage cells from seven different donors (age 30–54 years; mean 38 years) were evaluated for cell surface protein expression using flow cytometry. Three of these donors corresponded to the three samples evaluated by gene array.

Flow cytometry was performed on a Becton, Dickinson FACS Calibur (Franklin Lakes, NJ, http://www.bd.com/) using a 488-nm argon-ion laser for excitation; fluorescence emission was collected using 530/30 nm (FL1) and 585/42 (FL2) bandpass filters and logarithmic amplification. Cells were released with trypsin-EDTA and resuspended in DMEM/F12. The cells were then centrifuged and resuspended in wash flow buffer at a concentration of 1×10^6 cells/ml. Wash flow buffer consisted of phosphate buffer supplemented with 2% (v/v) FBS (Sigma) and 0.1% (w/v) sodium azide, NaN3 (Sigma). Cell viability was > 98% by the Trypan Blue dye (Gibco) exclusion technique. About $3–5 \times 10^5$ cells were stained with saturating concentrations of phycoerythrin (PE)–conjugated antibodies and isotype-matched controls. The cells were incubated in the dark for 30 minutes at 40°C. After incubation, cells were washed three times with wash flow buffer and resuspended in 0.25 ml of cold, protein-free phosphate-buffered solution (PBS) with 0.25 ml cold formaldehyde (2%) solution to preserve.

Flow cytometer instruments were set using unstained cells. Cells were gated by forward versus side scatter to eliminate debris. Since highly autofluorescent cells can overlap with cells expressing low levels of an antigen, the sensitivity of the PE signal was increased by eliminating the autofluorescence signal out of the FL1 channel and

thereby removing the contribution of autofluorescence in the measurement channel. A region (R2) was established to define positive PE fluorescence using a PE-conjugated isotype-matched control (Fig. 1). The number of cells staining positive for a given marker was determined by the percentage of cells present within a gate established such that fewer than 2% of the positive events measured represented nonspecific binding by the PE-conjugated isotype-matched control (Fig. 1). A minimum of 10,000 events was counted for each analysis.

For ABCG2 analysis, the A549 cell line (American Type Culture Collection [ATCC] no. CCL-185) was used as a positive control [21].

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Human ADAS cells were analyzed for telomerase and ABCG2 using RT-PCR techniques. Telomerase RT-PCR was performed with a Telomerase PCR Kit (Roche, cat. no. 1854666) according to manufacturer's instructions using a Bio-Rad iCycler PCR machine and a UVMax Kinetic Microplate Reader. Hela cells were used as positive controls, and Hela cells with Rnase were used as negative controls. A total of eight hADAS cell samples were tested.

Human ADAS cells from four patients were also screened for ABCG2 gene expression by RT-PCR. Confluent plates of hADAS cells were washed with PBS to remove debris and media. RNA was extracted from washed plates with adherent cells using an RNA extraction kit (Biotecx) per manufacturer's instructions. Briefly, cells were lysed in Ultraspec reagent. Equal volumes of chloroform were added and allowed to sit at 4°C for 15 minutes. The mixture was centrifuged at 14,000 G for 15 minutes. The top aqueous layer was removed and placed into a new tube. An equal volume of isopropyl alcohol was added and vortexed. RNA-Tack resin was added to bind free RNA in solution. Resin was vortexed, centrifuged into a pellet, washed with 70% ethyl alcohol (EtOH) twice, and then dried. Resin was resuspended in water to release bound RNA. The A260:280 ratio was obtained to determine quantity and purity. RNA was then converted to cDNA through reverse transcription using these reagent volumes in a 20-mL reaction volume: 1× 1st strand buffer; 0.5 mL RNasin (ribonuclease inhibitor); 0.5 mg poly-T primer; 2 mg bovine serum albumin (BSA); 1 mM dithiothreitol (DTT); 500 nM deoxynucleotide triphosphates (dNTPs); 50 U SuperScript II; and 1 mg mRNA. Reaction conditions were 10 minutes at 23°C, 60 minutes at 42°C, and 10 minutes at 94°C.

The synthesized cDNA was used to perform PCR to determine the presence or absence of ABCG2 transcripts. PCR was carried out using a BioRad iCycler PCR machine. The reaction mixture consisted of Invitrogen QPCR master mix (3 mM Mg^{2+}), SYBR Green, fluorescein, appropriate forward and reverse primers (500 nM each), deionized water (H_2O), and 5 mL cDNA. ABCG2 gene sequences used were forward primer (21-mer) 916 tcttctccattcatcagcctc 936, and reverse primer (21-mer) 1281 tcttcttcttcttctcacccc 1261, with an expected product of 366 bp. The A549 cell line was used as a positive control.

Results

Qualitative Confirmation of Developmental Plasticity

Using the qualitative assays described, the hADAS cell populations used in these studies differentiated in vitro into adipogenic, osteogenic, and neurogenic lineages (data not included). Adipogenic differentiation was considered positive based on a rounded morphology with intracellular lipid accumulation that stained with Oil Red O. Osteogenic differentiation was considered positive based on the appearance of nodules that stained positive for calcified matrix using von

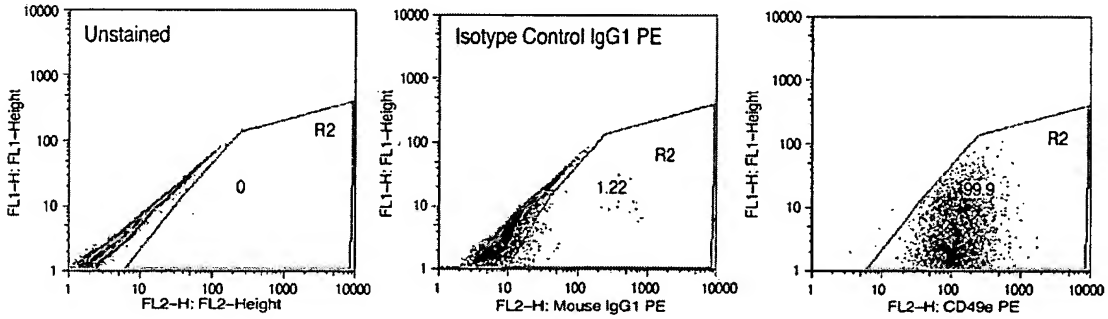

Figure 1. Two-parameter dot plots were used to display fluorescence data. An analysis region (R2) was established using a PE–conjugated isotype-matched control to contain <2% positive events. **(A)**: Unstained cells. **(B)**: Cells stained with isotype control. **(C)**: Cells stained with anti-CD49e PE. Abbreviation: PE, phycoerythrin.

Kossa staining. Neurogenic differentiation was considered positive based on cell morphology and positive staining for neurofilament β-tubulin.

Gene Array Analysis

Transcriptional characterization of three different hADAS cell populations was performed using microarrays for human extracellular matrix or adhesion molecules (SuperArray, cat. no. HS-010N) and human angiogenesis-related factors (cat. no. HS-009N). A representative array result is shown in Figure 2. Expected results were observed for internal controls on each array. Out of a total of 172 unique genes screened, only 7 genes were not transcribed by any of the hADAS cell populations. These unexpressed genes include the integrins αL, αM, β6, β7, selectin-L, and selectin-P and the apoptosis-related cysteine protease caspase 9. Of the 165 genes transcribed by at least one population, 114 (69%) were unanimously transcribed by all three hADAS cell populations, and a total of 143 (87%) were transcribed by at least two of the three populations (Table 2; Fig. 3). Some of the most highly transcribed genes include endoglin; FGFs 2, 6, and 7; FGF receptor 3; neuropilin-1; integrins α5 and α11; integrin β1; TGF-β receptors 2 and 3; SPARC (osteonectin); osteopontin; fibronectin-1; VEGF-D; TNF-α; and (MMP2) gelatinase A (Table 2). Twenty-two (13%) of the transcribed genes were transcribed by only one hADAS cell population, and all correspond to a single donor (see "Patient A," Fig. 3).

Flow Analysis

Cells from seven separate donors were evaluated by flow cytometry for the expression of over 24 cell surface proteins that relate primarily to extracellular matrix interactions. Flow results are summarized in Table 3. Of the 26 proteins screened, 21 were associated with highly consistent patterns of expression among all cell populations tested. Proteins that were consistently expressed by a majority of hADAS cells (average 97% or more of cells) included HLA-ABC and CD29 (integrin β1), CD49e (integrin α5/VLA-5), CD51 (integrin αV), and CD90 (Thy-1). Proteins that showed a positive, but more variable, expression across the seven donor groups include CD49b (integrin α2/VLA-2), CD49d (integrin α4/VLA-4), CD61 (integrin β3), CD138 (syndecan-1), and CD140a (PDGFR-β). The remainder of screened proteins were consistently absent or expressed on only negligible numbers of cells, including HLA-DR,

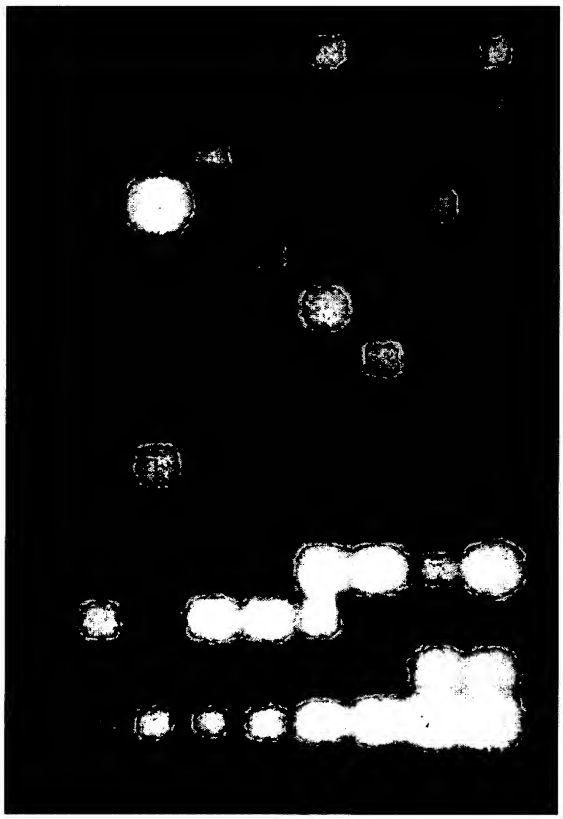

Figure 2. Representative example of a gene array expression profile of extracellular matrix and adhesion molecules for a human adipose-derived adherent stromal cell population. The bottom two rows include positive and negative controls.

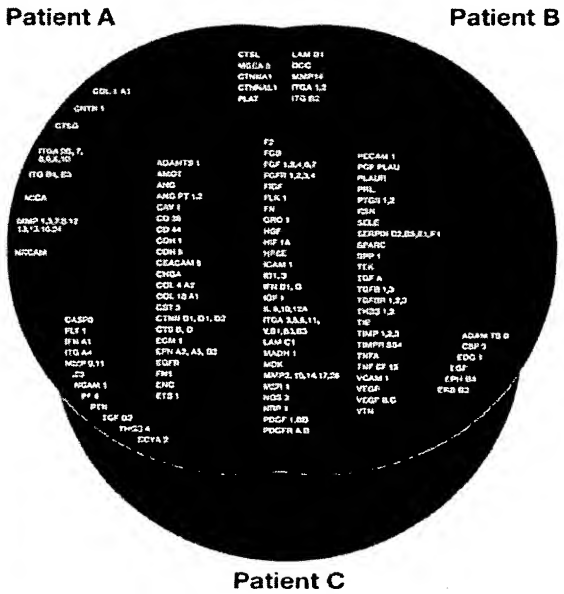

Figure 3. Venn diagram summarizing gene transcripts of all three human adipose-derived adherent stromal cell populations tested, as shown in Figure 2 (see also Table 2).

Table 2. Transcription profile of extracellular matrix and angiogenesis-related genes in early-passage, undifferentiated human adipose-derived adherent stromal (hADAS) cells ($n = 3$)

	CD Marker
Cell Adhesion Molecules	
Integrins:	
Integrin α3 (ITGA 3)	CD49c
Integrin α5 (ITGA 5); [fibronectin receptor][2–3x]	CD49e/VLA-5
Integrin α6 subunit (ITGA 6); [laminin receptor]	CD49f/VLA-6
Integrin αV (ITGA V)[1–2x]	
Integrin αV (ITGA V); [vitronectin receptor]	CD51
Integrin β1 (ITGB 1); [fibronectin receptor][1–2x]	CD29
Integrin β3 (ITGB 3); [gpIIIa]	CD61
Integrin β5 (ITGB 5)	
Integrin α1 (ITGA 1)[b]; [collagen and laminin receptor]	CD49a/VLA-1
Integrin α2 (ITGA 2)[b]	CD49b
Integrin α4 (ITGA 4)[b]	CD49d
Integrin β2 (ITGB 2)[b]	CD18
Integrin α10 (ITGA 10)[a]	
Integrin α2b (ITGA 2B)[b]	CD41b
Integrin α7 (ITGA 7)[b]	
Integrin α8 (ITGA 8)[b]	
Integrin α9 (ITGA 9)[b]	
Integrin αX (ITGA X)[b]	CD11c
Integrin β4 (ITGB 4)[b]	
Integrinβ8 (ITGB 8)[b]	
Cadherins and Catenins:	
Cadherin 1 type 1 (CDH 1); [E-cadherin (epithelial)]	
Cadherin 5 (CDH 5); [VE-cadherin (vascular epithelium)]	CD144
Catenin β1 (CTNNB 1)	
Catenin δ1 (CTNND 1)	
Catenin δ2 (CTNND 2); [cadherin-associated protein]	
Catenin α1 (CTNNA 1)[b]	
Catenin α-like 1[b] (CTNNAL 1)	
Other:	
GPIV (CD36)	CD36
H-CAM (CD44)	CD44
CEA (CEACAM 5)	CD66e
Endothelial leukocyte adhesion molecule I (SELE); [ELAM 1] CD62e	
Intercellular adhesion molecule 1 (ICAM 1)	CD54
Platelet/endothelial cell adhesion molecule (PECAM 1)	CD31
Vascular cell adhesion molecule 1 (VCAM 1)	CD106
Deleted in colorectal carcinoma (DCC)[b]	
Neural cell adhesion molecule 1 (NCAM 1)[b]	CD56
Contactin 1 (CNTN 1)[a]	
Neuronal cell adhesion molecule (NRCAM)[a]	
Matrix Proteins:	
Caveolin 1 (CAV 1)[1–2x]	
Collagen type IV α2 (COL4A 2)	
Collagen type 18 α1 (COL18A 1); [endostatin]	
Extracellular matrix protein 1 (ECM 1)	
Fibrinogen B (FGB)	
Fibronectin 1 (FN 1)[3–4x]	
Laminin γ1 (LAMC 1)[1–2x]	
Osteonectin (SPARC)[3–4x]	
Osteopontin (SPP 1); [bone sialoprotein I][3–4x]	
Thrombospondin 1 (THBS 1)[3–4x]	
Thrombospondin 2 (THBS 2)[3–4x]	
Thrombospondin 3 (THBS 3)	
Endoglin (ENG)[2–3x]	CD105
F 2, Human prothrombin (phil-3-clone)[2–3x]	
Restin (RSN); [Reed-Steinberg cell/intermediate filament-associated protein]	
Vitronectin (VTN); [serum spreading factor, somatomedin B]	
Laminin β1 chain (LAMB 1)[b]	
Thrombospondin-4 (THBS 4)[b]	
Collagen α1 (COLA 1)[a]	
MICA (MUC 18)[a]	CD146
Proteases	
Matrix Metalloproteinases:	
Metalloprotease/METH 1 (ADAMTS 1)	
Matrix metalloproteinase 2 (MMP 2); [gelatinase A, type IV collagenase][3–4x]	
Matrix metalloproteinase 10 (MMP 10); [stromelysin 2]	
Membrane-type matrix metalloproteinase 1 (MMP 14)	
Matrix metalloproteinase 17 (membrane-inserted) (MMP 17)	
Matrix metalloproteinase 26 (MMP 26)	
Human stromelysin-3 (MMP 11)[b]	
Matrix metalloproteinase 9 (MMP 9)[b]; [gelatinase B]	
Matrix metalloproteinase 20 (MMP 20)[b]; [enamelysin]	
Disintegrin-like metalloprotease (ADAMTS 8)[b]	
Matrix metalloproteinase 1 (MMP 1)[a]; [interstitial collagenase]	
Matrix metalloproteinase 3 (MMP 3)[a]; [stromelysin 1, progelatinase]	
Matrix metalloproteinase 7 (MMP 7)[a]	
Matrix metalloproteinase 8 (MMP 8)[a]; [neutrophil collagenase]	
Matrix metalloproteinase (MMP 12)[a]; [macrophage elastase]	
Matrix metalloproteinase 13 (MMP 13)[a]; [collagenase 3]	
Matrix metalloproteinase 15 (MMP 15)[a]	
Matrix metalloproteinase 16 (MMP 16)[a]	
Matrix metalloproteinase 24 (MMP 24)[a]	
Other:	
Cystatin C (CST 3)	
Cathepsin B (CTSB)	
Cathepsin D (CTSD)	
Heparinase (HPSE)	
Macrophage scavenger receptor 1 (MSR 1)	CD204
Plasminogen activator, urokinase (PLAU); [uPA]	
Prostaglandin-endoperoxide synthase 1 (PTGS 1); [Cox-1]	
Prostaglandin-endoperoxide synthase 2 (PTGS 2); [Cox-2]	
Urokinase-type plasminogen activator receptor (PLAUR)	
Transmembrane protease serine 4 (TMPRSS 4)	
Cathepsin L (CTSL)[b]	
Caspase 8 (CASP 8)[b]	
Meningioma expressed antigen 5 (hyaluronidase) (MGEA 5)[b]	
Plasminogen activator (PLAT)[b]	
Cathepsin G (CTSG)[a]	
Protease Inhibitors	
Plasminogen activator inhibitor, type I (SERPINE 1)[1–2x]	
Plasminogen activator inhibitor (SERPIN B2); [PAI-2]	
Protease inhibitor 5 (SERPIN B5)	
Pigment epithelium-derived factor (SERPINF 1)	
Tissue inhibitor of metalloproteinase 1 (TIMP 1)[3–4x]	
Tissue inhibitor of metalloproteinase 2 (TIMP 2)[3–4x]	
Tissue inhibitor of metalloproteinase 3 (TIMP 3)	
Growth Factors and Receptors	
Ephrin Family:	
Ephrin-A2 (EFNA 2)	
Ephrin-B2 (EFNB 2)	
Ephrin-A5 (EFNA 5)	
Eph B4 (EPHB 4)[b]	

(continued)

Table 2. Continued

Fibroblast Growth Factors and Receptors:
- Fibroblast growth factor 1 (FGF 1); [acidic]
- *Fibroblast growth factor 2 (FGF 2); [basic]*[2–3x]
- Fibroblast growth factor 4 (FGF 4)
- *Fibroblast growth factor 6 (FGF 6)*[2–3x]
- *Fibroblast growth factor 7 (FGF 7); [keratinocyte growth factor]*[2–3x]
- Fibroblast growth factor receptor 1 (FGFR 1)
- Fibroblast growth factor receptor 2 (FGFR 2)
- Fibroblast growth factor receptor 3 (FGFR 3)
- Fibroblast growth factor receptor 4 (FGFR 4)

Platelet-derived Growth Factors and Receptors:
- Platelet-derived growth factor α (PDGFA)
- Platelet-derived growth factor-BB (PDGF-BB); [PDGFb]
- Platelet-derived growth factor receptor α(PDGFRA) CD140a
- Platelet-derived growth factor receptor β (PDGFRB) CD140b
- Platelet factor 4 (PF4)[b]

Transforming Growth Factors and Receptors:
- Transforming growth factor α (TGF-A)
- Transforming growth factor β1 (TGF-B1)
- Transforming growth factor β3 (TGF-B3)
- Transforming growth factor β receptor I (TGF-BR1)
- *Transforming growth factor β receptor II (TGF-BR2)*[2–3x]
- *Transforming growth factor β receptor III (TGF-BR3)*[3–4x]
- Transforming growth factor β2 (TGF-B2)[b]

Vascular Endothelial Growth Factors and Receptors:
- *Vascular endothelial growth factor D (FIGF)*[3–4x]
- Placental growth factor (PGF); [vascular endothelial growth factor-related protein]
- Vascular endothelial growth factor (VEGF)
- Vascular endothelial growth factor B (VEGFB)
- Kinase insert domain receptor (FLK 1)
- TEK tyrosine kinase, endothelial (TEK)
- Tyrosine kinase with immunoglobulin and EGF homology domains (TIE)
- Vascular endothelial growth factor C (VEGFC)
- Vascular endothelial growth factor receptor (FLT 1)[b]

Others:
- Angiogenin (ANG)
- Angiopoietin-1 (ANGPT 1)
- Angiopoietin-2 (ANGPT 2)
- Angiostatin binding protein 1 (AMOT)
- Chromogranin A (CHGA); [parathyroid secretory protein 1, precursor for vasostatin]
- Epidermal growth factor receptor (EGFR)
- Hepatocyte growth factor (HGF); [hepapoietin A; scatter factor]
- Insulin-like growth factor 1 (IGF 1); [somatomedin C]
- Melanoma growth stimulating activity α (GRO 1)
- Nitric oxide synthase 3 (NOS 3)
- Endothelial differentiation sphingolipid G-protein-coupled receptor 1 (EDG 1)[b]
- Epidermal growth factor (EGF)[b]

Cytokines and Chemokines
- Interferon β1 (IFNB 1)
- Interferon γ (IFNG)
- Interleukin 8 (IL 8)
- Interleukin 10 (IL 10)
- Interleukin 12A (IL 12A)
- Midkine (MDK); [neurite growth-promoting factor 2]
- *Neuropilin 1 (NRP 1)*[2–3x]
- Prolactin (PRL)
- Tumor necrosis factor (TNFA); [TNF superfamily, member 2][3–4x]
- Vascular endothelial cell growth inhibitor (TNFSF 15)
- Colony stimulating factor 3 (CSF 3)[b]; [granulocyte]
- Interferon α1 (IFNA 1)[b]
- Pleiotrophin (PTN); [heparin binding growth factor 8/neurite growth-promoting factor 1][b]
- Small inducible cytokine A2 (SCYA 2)[b]

Transcription Factors
- DNA-binding protein inhibitor (ID 1)
- *Inhibitor of DNA binding 3 (ID 3); [dominant negative helix-loop-helix protein]*[3–4x]
- Mothers against decapentaplegic, *Drosophila* homolog 1 (MADH 1)
- *V-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS 1)*[2–3x]
- *Hypoxia-inducible factor 1 (HIF 1A); [basic helix-loop-helix transcription factor]*[3–4x]
- V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERB B2)[b]

[a]Expressed by only one of three hADAS cell populations analyzed.
[b]Expressed by two of three hADAS cell populations analyzed.
Italics indicate the highest expression levels relative to the cyclophilin-A housekeeping control gene. The relative level of expression is shown as superscript numbers (e.g., [3–4x]).

CD4, CD8a, CD11a (integrin αL), CD11b (integrin αM), CD11c (integrin αX), CD18 (integrin β2), CD41a (gpIIb), CD49f (integrin α6/VLA-6), CD62L (L-selectin), CD62P (P-selectin), CD106 (VCAM-1), CD117 (c-kit), CD133, CD243 (MDR-1), and ABCG2 (Table 3). Many of these cell surface proteins were also evaluated on a transcriptional level by microarray. Data summarizing both the transcriptional levels and the expression levels of these proteins are shown in Figure 4 and Table 3.

RT-PCR Analysis

Multiple hADAS cell populations were assayed by PCR for transcriptional evidence of two genes associated with a stem cell phenotype: telomerase and ABCG2. Our results revealed no evidence of either of these genes in hADAS cells that were cultured according to our methods (Fig. 5). The negative result for ABCG2 transcript correlates with a lack of detection of ABCG2 protein by flow cytometry (Table 3).

Discussion

In the study reported here, we established an extracellular matrix and angiogenesis-related transcriptional profile for early-passage, undifferentiated human adipose-derived adherent stromal (hADAS) cells. Isolated cell populations demonstrated multilineage developmental potential in vitro when exposed to culture conditions and qualitative assays well documented in the literature [1, 5, 20]. While it is recognized that these assays are neither wholly lineage-specific nor exhaustive in their scope, they enable an expedient confirmation of plasticity, especially against the backdrop of existing literature.

Early-passage, undifferentiated hADAS cells transcribe a wide variety of genes related to angiogenesis and the extracellular matrix. Only 7 of 172 genes evaluated were *not* transcribed by any of the hADAS populations. Of the 165 genes that were transcribed by hADAS cells, 22 (13%) were transcribed by only one of the three populations evaluated. Most of these single-population transcripts include various integrins and matrix metalloproteinases (Fig. 3). Most of our transcriptional data, however, suggests a high degree of consistency between cell populations isolated from different donors. For example, 143 (87%) out of a total 165 genes were transcribed by at least two of the three populations, and over two-thirds of these (69%) were transcribed unanimously by all three populations. Most of these transcripts and genes can easily be characterized as those consistent with primitive mesenchymal cells and include various growth factors and receptors, as well as integrins, extracellular matrix (ECM) proteins, and proteases, all of which are implicated in inflammation, matrix remodeling, angiogenesis, embryogenesis, organogenesis, tissue repair, and wound healing.

Of the 114 genes unanimously transcribed by early-passage, undifferentiated hADAS cells, those with the highest transcription levels include endoglin; FGFs 2, 6, and 7; FGF receptor 3; neuropilin-1; integrins α5 and α11; integrin β1; TGF-β receptors 2 and 3; SPARC (osteonectin); osteopontin; fibronectin-1; VEGF-D; TNF-α; and (MMP2) gelatinase A (Table 2). Expression levels were calculated relative to the positive control gene cyclophilin-A, with levels ranging from one to four times the level of cyclophilin-A. As a reference, Silva et al. [22] demonstrated that cyclophilin-A is the 15th most abundant transcript found in human marrow-derived stromal cells. In fact, many of the genes we found to be highly transcribed by hADAS cells are identical to the most abundant transcripts detected in human marrow-derived stromal cells [9, 22]. For example, SPARC, MMP 2, TIMP 1 and 3, fibronectin-1, integrin αV, integrin β1, and TGF-β are all highly abundant transcripts found in human marrow-derived stromal cells, as well as hADAS cells. Though limited to only 172 genes, our gene array data suggest a reproducible and consistent molecular profile among hADAS populations derived from different donors, as well as notable similarities to marrow-derived stromal cells.

This study also analyzed hADAS cells for the expression of over 24 cell surface proteins using flow cytometry. As summarized in Table 3, hADAS cell populations (n = 7) expressed these cell surface proteins in three general patterns: (a) those expressed by nearly all cells (>95%), (b) those expressed by minimal cells (<5%), and (c) those expressed by widely variable numbers of cells between these two extremes. Based on the proteins evaluated in this study, hADAS cells have a predominantly consistent and reproducible expression pattern of cell surface markers. Those that display variable expression patterns between populations

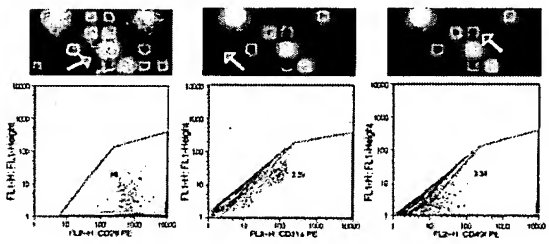

Figure 4. Representative gene array and flow cytometry data for integrin β1 (CD29), integrin αL (CD11a), and integrin α6 (CD49f). Pixels were quantitated using the GEArray Analyzer for each square present on the gene array. Genes were considered positive if signal intensity was greater than that of the negative control gene PUC18. Flow array charts show the percentage of cells expressing corresponding protein. The numbers in the dot plots represent raw counts and do not reflect nonspecific binding related to isotype-matched controls.

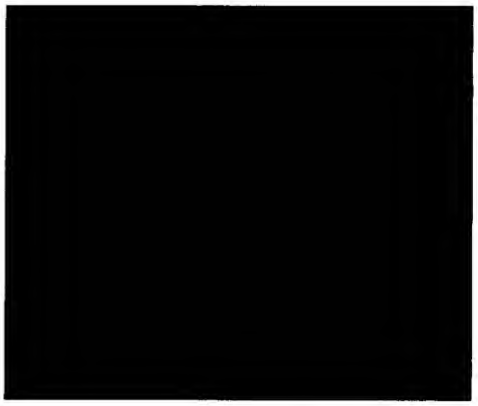

Figure 5. Gel results of ABCG2 reverse transcriptase polymerase chain reaction of human adipose-derived adherent stromal (hADAS) cells. The left lane is the A549 cell line (positive control) with a band between 300 and 400 bp (expected: 366 bp). The next four lanes are different hADAS cell populations. The right lane is a reference ladder in 100-bp increments.

(CD49b, CD49d, CD61, CD138, and CD140a) may reflect rapidly adapting cell responses to a dynamic extracellular milieu that is influenced by factors such as cell density, cell cycle, and time in culture.

Of note, the hADAS cell surface profile in this study corresponds excellently with published results from other groups. This is especially true on a qualitative level, and remains predominantly so when quantitative comparisons are made (Table 4) [2, 11, 12, 23]. In conjunction with our own internal population-to-population reproducibility, this uniformity with other groups' results suggests that adipose-derived stromal cell populations isolated on the basis of adherence to tissue culture plastic are perhaps more consistent and reproducible than has been recognized previously. This is particularly reassuring, given differences in nomenclature, subtle differences in cell isolation and culture, and differences in the materials and methods used for characterization between various groups. For example, the cells used in this study remained in culture twice as long before initial passaging as those used by Gronthos et al. [11], and subsequent passages were plated at a density of 2,000 cells/cm2 as compared to 10,000 cells/cm^2. In addition, Gronthos et al. used cryopreserved cells for some of their assays, whereas this study did not.

The effect, if any, on the cell surface profile and properties of hADAS cells due to these subtle differences is not currently known. However, quantitative differences in cell surface protein expression (example CD49d, CD49e) between our data and that of other groups may very well reflect differences in cell handling or cell detection, or both. Indeed, recent work suggests that the very exposure of adipose-derived SVF cells to culture plastic and their duration in culture (i.e., time on plastic) and the culture medium used significantly change the cell surface phenotypic profile

Table 3. Gene transcription and protein expression levels of select markers by early-passage, undifferentiated human adipose-derived adherent stromal cells

Protein	Transcription (gene array) $n=3$		Expression (flow)[a] $n=7$	
	Tx. level[b]	*(+) Population.*[c]	*Mean% ± SDs*	*Range (%)*
HLA-DR PE	NT	NT	1% ± 1	0–3
HLA-ABC PE	NT	NT	97 ± 2	95–99
CD4 (MHC class II co-receptor; T4)	NT	NT	1 ± 0.6	0.2–2
CD8a (MHC I co-receptor; T8)	NT	NT	0[d]	0
CD11a (integrin αL; LFA-1)	0	0/3	1 ± 1	0–2
CD11b (integrinαM; Mac-1)	0	0/3	1 ± 0.6	0.1–2
CD11c (integrin αX; p150; CR4)	0.2x	1/3	1 ± 0.8	0.1–2
CD18 (integrin β2)	0.025–0.4x	2/3	1 ± 0.5	0–1
CD29 (integrin β1)	1.1–1.4x	3/3	97 ± 1.5	95–99
CD41a (gpIIb)	NT	NT	0 ± 0.1	0–0.2
CD49b (integrin α2; VLA-2)	0–0.5x	2/3	80 ± 13	59–96
CD49d (integrin α4; VLA-4)	0–0.27x	2/3	78 ± 20	37–97
CD49e (integrin α5; VLA-5)	0.8–1.4x	3/3	98 ± 1	96–99
CD49f (integrinα6; VLA-6)	0.6–1.0x	3/3	1 ± 1	0–4
CD51 (integrin αV; vitronectin receptor)	0.4–0.9x	3/3	97 ± 3	92–100
CD61 (integrin β3; gpIIIa)	0.56x	3/3	30 ± 26	8–68
CD62L (L-selectin; LECAM-1)	0	0/3	0[d]	0
CD62P (P-selectin; PADGEM)	0	0/3	0[d]	0
CDw90 (Thy-1)	NT	NT	98 ± 1	96–100
CD106 (VCAM-1)	0.06–0.3x	3/3	1 ± 2	0–5
CD117 (c-kit; SCFR)	NT	NT	0.2 ± 0.4	0–1
CD133 (AC133)	NT	NT	1 ± 0.7	0–2
CD138 (Syndecan-1)	NT	NT	26 ± 17	10–54
CD140a (PDGFR-α)	1.4–3.0x	3/3	41 ± 26	12–86
CD243 (MDR-1; p170; P-gp)	NT	NT	0.05 ± 0.1	0–0.3
ABCG2	NT	NT	0.01 ± 0.02[e]	0–0.05

[a]Percentage of cells expressing protein, minimum 10,000 events counted.
[b]Transcription level relative to cyclophilin-A positive-control housekeeping gene.
[c]Number of hADAS cell populations demonstrating positive transcription.
[d]$n=6$.
[e]$n=8$ (cells from five different sources, ranging from primary plating, P0, to passage 10).
Abbreviation: NT, not tested.

of such cells (personal communications and [24, 25]). For example, freshly isolated human SVF cells demonstrate significant numbers of cells positive for hematopoietic lineages and endothelial cells (ex. CD45, CD14, CD144, CD34), which notably decrease after 3–5 days in culture [24, 25]. We have found similar profile changes before and after exposure to tissue culture plastic, as well as subtle but definite trends toward loss of differentiated phenotypes with extended time in culture and with increasing passage (unpublished results). This emerging picture is a primary reason why we incorporate "adherent" into the description of these cells, so as to distinguish them from freshly isolated (i.e., SVF) cells from adipose tissue which have *not* been exposed to plastic and which have a significantly different cell surface phenotypic profile. The relationship between variables such as plastic exposure, culture medium, culture duration, passage number, cryopreservation, harvest depot, and donor age to SVF and hADAS cell phenotypic profile and developmental plasticity certainly deserves further investigation.

Just as hADAS cells have many similarities with MSCs on a transcriptional level, they are also quite similar on a cell surface protein level. As summarized in Table 4, when compared with published reports for MSCs, hADAS cells share a great majority of cell surface expression patterns, at least from a qualitative perspective. Our data do suggest a few differences (such as CD49d, CD62L, and CD106), which are consistent with reports from other groups [2, 11, 12]. However, these same markers have been a source of discrepant findings within the MSC literature itself [26–28]. Without quantitative parameters, however, it is hard to assess the extent and significance of such differences. Nevertheless, the current transcriptional and cell surface profile of hADAS cells is strikingly similar to adherent stromal cells derived from the bone marrow (i.e., MSCs). Moreover, published reports of developmental plasticity are undeniably similar between the two groups of cells. Further work is necessary to delineate the extent and significance of any differences that exist between these two cell types and tissue sources.

Many of the CD markers that were screened in this study by flow cytometry were chosen to correlate with adhesion molecule genes evaluated by gene array. Results of this dual-level evaluation of these particular factors are summarized in Table 3. In addition, some of the proteins evaluated in this study were chosen because of their correlation to "stemness" in other stem cell populations. For example, CD133 is associated with the prospective identification

Table 4. Literature review of cell surface phenotype of human adipose-derived adherent stromal (hADAS) cells and comparison to published results for human marrow-derived mesenchymal stem cells (MSCs); percentages given where available

Cell surface marker	Human ADAS cells		Human MSCs
	Katz et al. [xx] $n=7$	Gronthos et al. [11] $n=3–5$	Others	Various Authors
CD4/MHC class II co-rec.,T4	1%	NA	NA	–[27]
CD11a/integrin αL; LFA-1	1%	0	NA	–[27]
CD11b/integrin αM	<1%	1%	–[23]	NA
CD11c/integrin αX	1%	0	NA	NA
CD18/integrin 2	<1%	0	NA	–[27]
CD29/integrin β1	97%	98%	+[2, 12, 23]	+[2, 12, 22, 27]
CD31	NA	1%	–	–[2]
CD44	NA	60%	+[2, 12, 23]	+[2, 12, 22, 27]/(100%) [9]
CD49b/integrin α2	80%	NA	NA	+[27]
CD49d/integrinα4	78%	9%	+[2, 12]	–,[2, 12, 22, 27]
CD49e/integrinα5	98%	22%	+[23]	+[22, 27]
CD51/integrin αV	97%	NA	NA	+[27]/–[22]
CD61/integrin β3	30%	NA	–[2]	+[27]/–[22]5
CD62e	NA	2%	–[2]	–[27]
CD62L/L-selectin	0	NA	NA	+[27]
CD62P/P-selectin	0	NA	NA	–[27]
CDw90/Thy-1	98%	NA	+[2, 12, 23]	+[2, 12, 22, 27]/(90%) [9]
CD105 (SH2)/endoglin	NA	36%	+[2, 12]	+[2, 12]
CD106/VCAM-1	1%	+[a]	–[2, 12]	+,[2, 12, 27]
CD117/c-kit; SCFR)	0.3%	NA	NA	NA
CD133/AC133	1%	NA	NA	NA
CD140a/PDGFR-α	41%	NA	NA	+[27]
HLA-ABC	97%	93%	+[23]	+[22]
HLA-DR	<1%	–	[23]	–[22]

See the reference list for the sources in brackets.
[a]Immunohistochemistry
Abbreviations: NA, not available.

and isolation of hematopoietic stem cells (HSCs) [18, 29, 30]. As noted in Table 3, CD133 was detected on minimal, if any, hADAS cells from the seven populations tested in this study. Similarly, ABCG2 is a member of the ATP binding cassette (ABC) superfamily of membrane transporters whose expression has recently been shown to be a conserved feature of stem cells from a wide variety of sources. It has recently been shown to confer the side-population phenotype which correlates with a stem cell population within bone marrow specimens [16, 17, 21, 31]. Forced expression of ABCG2 directly confers a stem cell–like phenotype to bone marrow cells; and, like telomerase, ABCG2 expression is downregulated with differentiation. For these reasons, ABCG2 has been postulated as a molecular marker of stem cell phenotype. Our results demonstrate that this putative stem cell marker is not expressed on the surface of hADAS cells. Because the number of cells anticipated to express this marker is extremely low, and because our flow data ambiguously fit into this potential range, we confirmed our flow results with RT-PCR. The flow and PCR results together suggest that hADAS cells do not express the ABCG2 protein (Table 3; Fig. 5).

Finally, our findings suggest that hADAS cells do not express the ribonucleoprotein telomerase, as determined by RT-PCR (data not shown). Telomerase prevents telomeric shortening and correlates with extended replicative capacity in certain tumor cells and stem cells, and its expression correlates to the undifferentiated state [13, 15, 32]. Our negative findings, however, are similar to recent work that demonstrates a lack or absence of telomerase in cell populations that are otherwise considered stem cell candidates [33, 34].

Summary

Early-passage, undifferentiated hADAS cells transcribe many genes that are related to the extracellular matrix and angiogenesis and which are implicated in matrix remodeling, inflammation, morphogenesis, and tissue repair. Highly transcribed genes include endoglin; FGFs 2, 6 and 7; FGFR3; neuropilin-1; osteonectin; fibronectin; VEGF-D; TGF-β R2 and R3; and integrins α5, α11, and β1. Our findings suggest remarkably good consistency in the transcriptional profile of hADAS cells isolated from different donors, as well as many similarities with published profiles for marrow-derived stromal cells. This study also confirms and expands on the cell surface profile of hADAS cells and confirms many cell surface similarities with marrow-derived stromal cells. More specifically, our results demonstrate good donor-to-donor consistency and reproducibility, both internally and when compared to previously published data from other groups. This uniformity is remarkable, given the inconsistent nomenclature and nonstandardized cell isolation and culture protocols. Of note, emerging evidence suggests that both adherence to plastic and time in culture appear to alter the cell surface phenotype. Our findings support the notion that adipose-derived stromal cells isolated by adherence to tissue culture plastic have a remarkably consistent molecular and cell surface profile, yet lack an easily definable phenotype. In the near future, it is hoped that a consensus on nomenclature and hADAS cell isolation and culture protocols will emerge, so as to allow more efficient and meaningful communication and interpretation of published research. Continued characterization of these cells will also help clarify phenotypic, developmental, and regenerative differences between them and freshly isolated SVF cells, and it may ultimately enable the *prospective isolation* of specific, purified subpopulations of putative multipotential stem cells and lineage-committed progenitor cells.

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Cell Surface and Transcriptional Characterization of Human Adipose-Derived Adherent Stromal (hADAS) Cells

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